Neonatal Imprinting and Hepatic Cytochrome P-450 II. Partial Purification of a Sex-Dependent and Neonatally Imprinted Form(s) of Cytochrome P-450

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A new form of cytochrome P-450 was partially purified from hepatic microsomes of neonatally imprinted rats (adult male and adult male castrated at four weeks of age). This new form of cytochrome P-450 appears to have an apparent molecular weight of approximately 50,000 daltons as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It appears that this form of cytochrome P-450 is either absent or present in low concentrations in cytochrome P-450 preparations isolated from neonatally nonimprinted rats (adult female and adult male castrated at birth). Reconstitution of testosterone hydroxylase and benzphetamine N-demethylase activities of this partially purified cytochrome P-450 revealed that the presence of testosterone 16α-hydroxylase activity, an imprintable microsomal enzyme, was in parallel with the imprinting status of the animals; a significantly higher activity was detected in the neonatally imprinted than that of the nonimprinted animals. This was in contrast to the nonimprintable benzphetamine N-demethylase, testosterone 7α- and 6β-hydroxylase activities which exhibited no correlation with the imprinting status of the animals.

We have prepared antisera from rabbits using the partially purified cytochrome P-450 preparations from adult male rats as antigens. These antisera inhibited microsomal testosterone 16α- and 7α-hydroxylase activities in a concentration-dependent manner, without impairing 6β-hydroxylase activity. These data suggest that the partially purified cytochrome P-450 from adult male rats consists of both imprintable (16α-) and nonimprintable (7α-) testosterone hydroxylase activities. The antisera formed immunoprecipitant lines in the Ouchterlony double diffusion plates with partially purified cytochrome P-450 from both neonatally imprinted and nonimprinted adult rats. The immunoprecipitant lines, as stained by coomassie blue, suggest the homology of the cytochrome P-450 preparations from neonatally imprinted and nonimprinted rats. Immunoabsorption of the antisera against neonatally nonimprinted, partially purified cytochrome P-450 completely removed the immunoprecipitant lines without appreciably impairing the inhibitory effects of antisera on the microsomal testosterone 16α- and 7α-hydroxylase activities. In contrast, immunoabsorption of the antisera against partially purified cytochrome P-450 from adult male rats (imprinted) abolished completely both the immunoprecipitant lines and the inhibition on microsomal testosterone hydroxylation reaction (16α and 7α). The

inhibitory action of antisera on testosterone hydroxylation was also abolished
upon boiling the antisera at 100°C for 5 minutes.

The biochemical and immunochemical data in this study suggest that the
neonatally imprintable form or forms of hepatic microsomal cytochrome P-
450 accounts for a small fraction of the bulk of total cytochrome P-450. How-
ever, the existence of this form of cytochrome P-450 is regulated by gonadal
hormones during the neonatal period and accounts for the major imprintable
sex differences in drug and steroid metabolism in adulthood.

Although certain sex-dependent differences in the oxidative metabolism of steroids
and drugs are known to be imprinted during the neonatal period [1–4], the exact site(s)
of such imprinting have not yet been resolved. Since the monooxygenation of steroids
or drugs in the microsomal membranes is determined by the reactive components such as
the NADPH-cytochrome c reductase, cytochrome P-450, and the lipid, alterations in
either one or all of these constituents can result in marked differences in substrate specific-
ity. We have demonstrated previously that testosterone 16α-hydroxylase activity exists
predominantly in one of the cytochrome P-450 fractions (peak-II) eluted from a DEAE-
cellulose column. This form or forms of cytochrome P-450 is/are regulated by neonatal
androgen imprinting [5]. In the present study, we report the purification of this form of
cytochrome P-450 from adult male rats. The catalytic activities and electrophoretic prop-
erty of this partially purified form of cytochrome P-450 were compared to cytochrome
P-450 similarly purified from other neonatally imprinted and nonimprinted rats. In the
present report, we have obtained biochemical and immunochemical evidence to suggest
that this neonatally imprinted form of cytochrome P-450 only represents an extremely
small fraction of the bulk of total hepatic microsomal cytochrome P-450. This small frac-
tion of cytochrome P-450, however, accounts for the bulk of the neonatally androgen-
imprinted differences in testosterone hydroxylation in hepatic microsomes in adulthood.

MATERIALS AND METHODS

Adult Wistar rats (200–300 gm) were purchased from Microbiological Associates,
Bethesda, MD. They were maintained on cottonwood shavings and Purina Laboratory
Chow and water ad libitum. Newborn litters were derived from random mating pairs
which consisted of one male and two or three females. Rats were routinely weaned at the
age of 21–23 days. In some experiments, rats were castrated either at birth or at the age
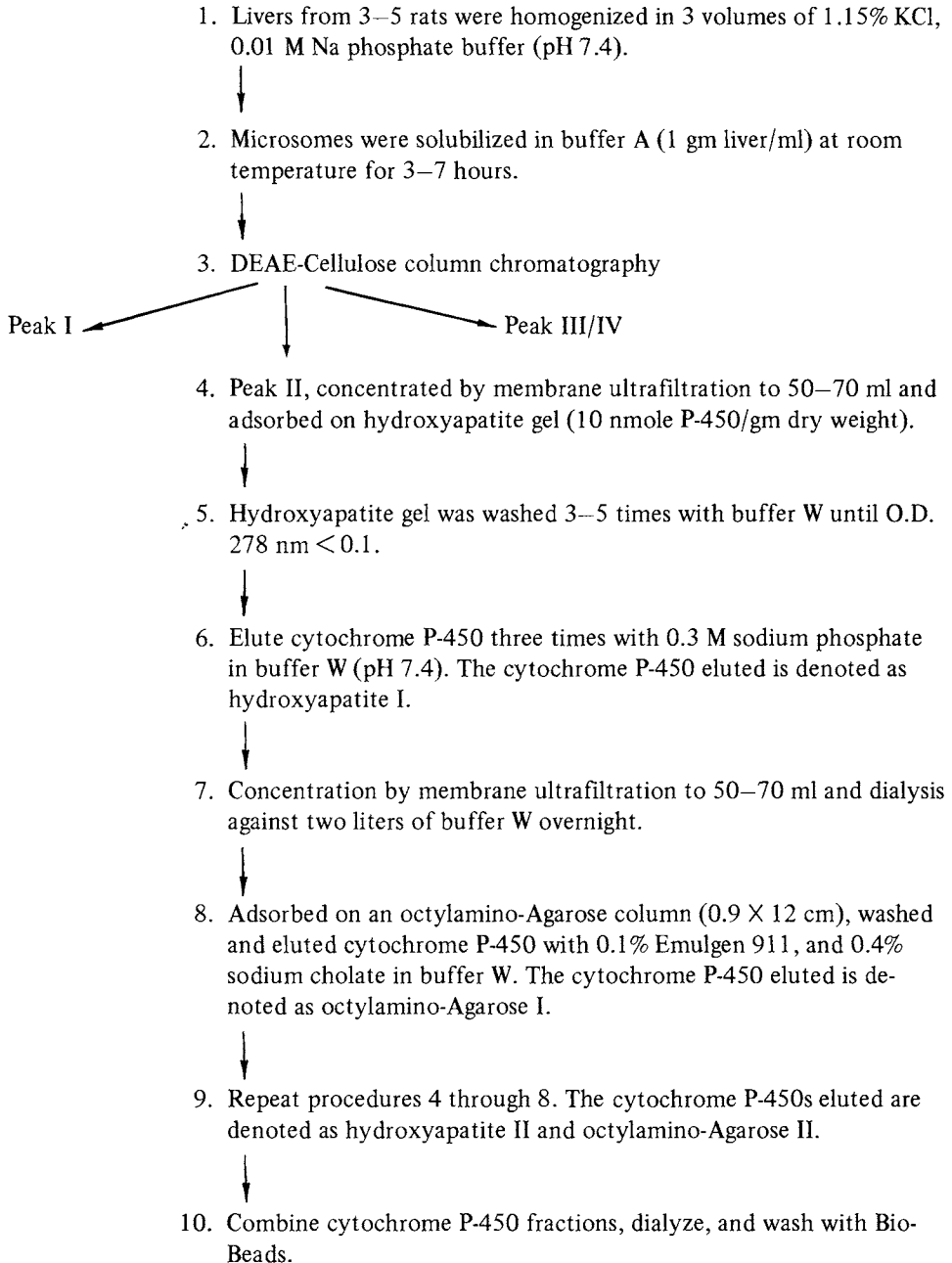of four weeks according to the procedures previously described [4].

Antisera against the partially purified cytochrome P-450 from adult male rat micro-
somes were prepared according to the method described by Vaitukaitis et al [23]. In
brief, adult New Zealand white female rabbits were immunized with the partially purified
cytochrome P-450 from adult male rats via subcutaneous and intradermal routes at mul-
tiple sites (0.1 ml/injection for a total of 2.5–3 ml) on the back of both sides of the spinal
cord. Rabbits were injected first with the antigen emulsified with the complete Freund's
adjuvant (cytochrome P-450 diluted with 0.9% NaCl and mixed 1:1 with complete
Freund's adjuvant). This was followed by two subsequent injections of the antigen emul-
sified with the incomplete Freund's adjuvant at two weeks' interval. Seven to nine days
after the last injection, rabbits were bled for antisera via ear veins. Each rabbit received a

total of 280 μg of cytochrome P-450 (200, 40, 40 μg for the first, second, and third immunization, respectively).

Antisera were analyzed by both Ouchterlony double diffusion plates (see legend to Figure 2) and the inhibition of testosterone hydroxylation in a microsomal assay system [5]. In brief, microsomes were isolated from hepatic tissues of normal adult male rats by homogenization and ultracentrifugation techniques [4]. Microsomes were resuspended in 1.15% KCl–0.01 M phosphate buffer (pH 7.4) at a ratio of 1 gm of liver per ml. The assay system consists of 1,2-(^{14}C)-testosterone (40 nmole, 46 mCi/ml), MgCl$_2$ (2.5 μmole), sodium phosphate (22.5 μmole), NADPH (0.25 μmole), and cytochrome P-450 in microsomal suspensions (0.05 nmole) in a final volume of 0.25 ml. This assay condition has been established previously as optimal with respect to cytochrome P-450 concentration [5].

Partial Purification of a Sex-Dependent, Neonatally Imprinted Form of Hepatic Cytochrome P-450

Unless otherwise indicated, all isolation procedures were conducted at 2°C. The individual purification steps are depicted in Scheme 1. Livers from 3–5 rats were homogenized in three volumes of 1.15% (w/v) KCl, 0.01 M sodium phosphate (pH 7.4). Microsomes were isolated and resuspended in buffer A (10 mM Na phosphate, 0.5% (w/v) cholic acid, 0.2% (w/v) Emulgen 911 (Kao Atlas, Tokyo), 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol, pH 7.4) at room temperature for 3–7 hours at a ratio of 1 gm/ml as described earlier [5]. The solubilized microsomes were fractionated by DEAE-cellulose column chromatography (DE-52, Whatman, England) at room temperature (step 3). The various fractions collected under peak II were combined and concentrated by membrane ultrafiltration (Amicon, step 4) to approximately 50–70 ml. The concentrated peak II was adsorbed onto a slurry of hydroxyapatite gel (Bio-Rad, Richmond, CA), previously equilibrated with buffer A at a ratio of 10 nmole P-450/gm dry weight (step 4). This was accomplished by occasional stirring of the gel containing cytochrome P-450 fraction for a period of 15 minutes. Over 95% of the cytochrome P-450 was adsorbed onto the hydroxyapatite gel under these conditions. The gel was then washed 3–5 times with buffer W (10 mM Na phosphate, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol, pH 7.4) until the optical density of 278 nm was less than 0.1 (step 5). Cytochrome P-450 was then eluted three times from the gel with buffer W containing 0.3 M sodium phosphate (approximately 150–200 ml, step 6). The eluted P-450 fraction was then concentrated by membrane ultrafiltration to approximately 50–70 ml and dialyzed overnight against two liters of buffer W (step 7). The cytochrome P-450 was then fractionated by an affinity hydrophobic column, octylamino-Agarose (P-L Biochem., Milwaukee, WI) according to the procedures described by Imai and Sato ([7], step 8). The column was pre-equilibrated with buffer W and the sample loaded at a flow rate of approximately 0.5 ml/minute. The column was then washed with 25 ml of buffer W. Cytochrome P-450 was eluted from the column with 50–100 ml of buffer W containing 0.1% (w/v) Emulgen 911 and 0.4% (w/v) decolorized and recrystallized cholic acid ([7], step 8). For further purification, steps 4 through 8 were repeated. The final partially purified cytochrome P-450 was dialyzed overnight against 50–100 volumes of buffer W to remove cholic acid. The residual Emulgen 911 was removed by 3–4 washes with Bio-Beads (SM-2, Bio-Rad, Richmond, CA) [5]. The concentration of Emulgen 911 in the washed samples was estimated to be <0.01% (w/v). These detergent-depleted samples were then used for both reconstitution studies and polyacrylamide gel electrophoresis analysis.

Scheme 1. Partial Purification of Hepatic Cytochrome P-450 From Rat Microsomes

1. Livers from 3–5 rats were homogenized in 3 volumes of 1.15% KCl, 0.01 M Na phosphate buffer (pH 7.4).

2. Microsomes were solubilized in buffer A (1 gm liver/ml) at room temperature for 3–7 hours.

3. DEAE-Cellulose column chromatography

Peak I ◄————— | —————► Peak III/IV

4. Peak II, concentrated by membrane ultrafiltration to 50–70 ml and adsorbed on hydroxyapatite gel (10 nmole P-450/gm dry weight).

5. Hydroxyapatite gel was washed 3–5 times with buffer W until O.D. 278 nm < 0.1.

6. Elute cytochrome P-450 three times with 0.3 M sodium phosphate in buffer W (pH 7.4). The cytochrome P-450 eluted is denoted as hydroxyapatite I.

7. Concentration by membrane ultrafiltration to 50–70 ml and dialysis against two liters of buffer W overnight.

8. Adsorbed on an octylamino-Agarose column (0.9 X 12 cm), washed and eluted cytochrome P-450 with 0.1% Emulgen 911, and 0.4% sodium cholate in buffer W. The cytochrome P-450 eluted is denoted as octylamino-Agarose I.

9. Repeat procedures 4 through 8. The cytochrome P-450s eluted are denoted as hydroxyapatite II and octylamino-Agarose II.

10. Combine cytochrome P-450 fractions, dialyze, and wash with Bio-Beads.

Reconstitution of Testosterone Hydroxylase and Benzphetamine N-Demethylase Activities

Testosterone hydroxylase and benzphetamine N-demethylase activities were reconstituted by the addition of the following components: Partially purified cytochrome P-450 (0.2 nmole); affinity column-purified NADPH-cytochrome c reductase (100 units, see [5]); dilauryl phosphatidyl choline (15 μg, Sedary Research Laboratory, London, Ontario); $MgCl_2$ (10 μmole); sodium phosphate (90 μmole); NADPH (1 μmole); and substrates (1,2-[^{14}C]-testosterone, 160 nmole, 46 mCi/mmole; or N-^{14}C-benzphetamine, 528 nmole, 0.14 mCi/mmole, kindly provided by Dr. J. G. Joly, Hospital St. Luc, Montreal, Quebec) in a final volume of 1 ml according to the general procedures outlined by Lu and Levin [8].

Testosterone hydroxylase activities were determined by the method previously described [5, 9]. In brief, incubation was allowed to proceed in air for 15 minutes at 35°C under gentle shaking in a Dubnoff incubator. The reaction was stopped by the addition of 5 ml of methylene chloride. Testosterone and its metabolites were then extracted from the aqueous phase into the organic phase by thorough mixing of both phases at room temperature for 30 minutes. The organic phase was removed, dried under nitrogen, and redissolved in ethyl acetate containing 20 μg each of the testosterone standards (16α, 2β, 7α and 6β). The samples were then spotted on plastic silica gel plates that were precoated with a fluorescent indicator (Eastman Kodak, Rochester, NY) and separated by the solvent system of chloroform:ethyl acetate:ethanol (4:1:0.4). The R_f values for 16α-, 2β-, 7α- and 6β-hydroxytestosterone were 0.32, 0.40, 0.45, and 0.61, respectively. Since only an insignificant amount of 2β-hydroxytestosterone was formed under the present assay conditions, this metabolite was ignored. The reference standards were detected by visualization under a UV light (254 nm), cut, and counted in 6 ml of PCS (Amersham/Searle, Des Plains, IL) by a Beckmann Scintillation Spectrophotometer (LS-150).

Benzphetamine N-demethylase activity was determined by the procedures described by Thomas et al [10] with slight modification. The reaction was terminated by the addition of 0.1 ml of 1 N NaOH at the end of 15 minutes incubation at 35°C. Unreacted benzphetamine was removed by organic solvent extractions (2.5 ml heptane followed by 2.5 ml chloroform). An aliquot of the aqueous phase containing H ^{14}CHO was removed and counted as described above.

Polyacrylamide Gel Electrophoresis

Partially purified cytochrome P-450 and representative samples from the various purification steps were subjected to sodium dodecyl sulfate (SDS) slab-gel electrophoretic analysis according to the procedures of Laemmli [11]. The final concentrations of acrylamide and SDS were 7.5% (w/v) and 0.1% (w/v), respectively. The protein markers used were bovine serum albumin (68,000 M.W.), glutamate dehydrogenase (53,000 M.W.), ovalbumin (45,000 M.W.), aldolase (40,000 M.W.), and chymotrysinogen A (25,000 M.W.)

Other Analytical Methods

Protein was determined by the method of Lowry et al [12] following perchloric acid precipitation (0.8 N) using bovine serum albumin as the reference standard. Cytochrome P-450 was determined by its reduced CO-difference spectrum according to the procedures of Omura and Sato [13]. NADPH-cytochrome c reductase was determined by the method described by Phillips and Langdon [14].

TABLE I. Partial Purification of Hepatic Cytochrome P-450 From the Peak II of DEAE-Cellulose Eluate of the Adult Untreated Male Rats*

Purification steps	Total cytochrome P-450 (nmoles)	Total protein content (mg)	Specific content (nmoles/mg)	Fold of purification	Recovery of cytochrome P-450 (%)
DEAE-Cellulose	277	176	1.57	1.0	100
Hydroxyapatite I	191	106	1.81	1.2	69
Octylamino-agarose I	128	48.1	2.65	1.7	46
Hydroxyapatite II	91.5	35.2	2.60	1.7	33
Octylamino-agarose II	25.2	3.50	7.20	4.6	9

*Partial purification steps are described in Scheme 1 in text. Typical results of isolation of a neonatally imprintable form or forms of cytochrome P-450 from adult untreated male rats were presented.

RESULTS

Using the isolation procedures depicted in Scheme 1, cytochrome P-450 was partially purified from the livers of adult untreated male rats (Table I). The specific content of the partially purified cytochrome P-450 preparation was 7.2 nmole/mg of protein. This represents a 4.6-fold purification of the peak II eluted from DEAE-cellulose column or a tenfold purification over that of the original liver homogenate [3, 15]. Total recovery of cytochrome P-450 was 9%. The hemoproteins were quite stable during purification, and no conversion to cytochrome P-420 was observed. Throughout the purification, neonatally imprinted testosterone 16α-hydrolase activity was retained and enriched. The specific activity of this enzyme activity in the final partially purified preparation was approximately five times that of the activity obtained from the peak II of the DEAE eluate (Table II). On the other hand, the specific activities of both 7α- and 6β-testosterone hydroxylases appeared to fluctuate during different purification steps. The final partially purified enzyme preparations still retained 7α- and 6β-hydroxylase activities.

This same procedure was also utilized to partially purify the cytochrome P-450 from peak II isolated from the neonatally imprinted (adult male castrated at four weeks of age) and nonimprinted (adult female and adult male castrated at birth) rats. The final specific contents of the partially purified P-450 preparations were 5.5, 6.7, and 4.6 nmole/ mg, respectively (Table III). Reconstitution of the enzyme activities of these partially purified cytochrome P-450 preparations revealed that the imprintable testosterone 16α-hydroxylase activity only exists in appreciable amounts in the adult male and male rats castrated at four weeks of age (imprinted), whereas adult female and male rats castrated at birth (nonimprinted) have much lower activity (Table III). In contrast, several other enzyme activities, 7α-, 6β-hydroxylases and benzphetamine N-demethylase, which are known not to be regulated by neonatal imprinting, failed to show a similar correlation with neonatal imprinting status of the animals (Table III).

These partially purified cytochrome P-450 preparations were also subjected to polyacrylamide gel electrophoretic analysis. Figure 1 shows the gel patterns of these preparations. There are two major protein bands in the cytochrome P-450 preparation from the adult male rats with molecular weights of approximately 50,000 and 52,000 daltons (track 5). The gel patterns of cytochrome P-450 preparations from the adult female (track 4), adult male castrated at four weeks of age (track 3), and adult male castrated at birth (track 2) were considerably more complex and contained many other proteins similar to those that exist in the semi-purified fractions (DEAE, hydroxyapatite, and octylamino-

TABLE II. Reconstituted Testosterone Hydroxylase Activities During Different Purification Steps of Cytochrome P-450 Isolated From the Peak II of Adult Untreated Male Rats

Purification steps[a]	Cytochrome P-450 specific content	Testosterone hydroxylase activities (nmoles product/0.2 nmole P-450/15 min)		
		16α	7α	6β
DEAE-Cellulose	1.57	0.82	0.41	0.32
Hydroxyapatite I	1.81	4.82	1.40	0.53
Octylamino-agarose I	2.65	4.84	2.35	2.65
Hydroxyapatite II	2.60	2.96	1.37	0.45
Octylamino-agarose II	7.20	4.37	0.47	2.30

[a]Cytochrome P-450 samples from each purification step were assayed for testosterone hydroxylase activities in a reconstituted system. Data represent the average of duplicate assays with variations between assays less than 5%.

TABLE III. Testosterone Hydroxylase and Benzphetamine N-Demethylase Activities in Partially Purified Cytochrome P-450 Isolated From Neonatally Imprinted and Nonimprinted Rats*

Partially purified cytochrome P-450	Cytochrome P-450, specific activity (nmole/mg)	Testosterone hydroxylase activities			Benzphetamine N-demethylase activities
		(nmole products formed/0.2 nmole P-450/15 min)			
		16α	7α	6β	
Adult male	7.2	9.36	1.41	0.71	20.35
Adult female	5.5	0.60	2.27	nil	12.25
Adult male castrated at birth	6.7	1.04	2.59	0.20	4.03
Adult male castrated at 4 weeks	4.6	4.21	1.90	0.39	7.02

*Cytochrome P-450s were partially purified from neonatally imprinted and nonimprinted rats. Enzyme activities were assayed in a reconstituted system according to the Methods, and were expressed as average of duplicate assays with variations between assays less than 5%.

Agarose fractions, tracks 8, 7, and 6, respectively). These differences in the complexities of gel patterns in the final partially purified P-450 were reflected in their differences in specific contents as indicated in Table III. When comparing the gel electrophoretic patterns of these partially purified cytochrome P-450 preparations, it appeared evident that one of the major protein bands in the adult male at 50,000 daltons (indicated by arrow) was very faint or absent in the adult female and adult male castrated at birth, but appeared prominent in adult male castrated at four weeks of age. This correlation in electrophoretic mobility of a form or forms of cytochrome P-450 with imprinting status of the animal was not demonstrated when compared to the total microsomal cytochrome P-450 between neonatally imprinted and nonimprinted rats.

   Since the partially purified cytochrome P-450 preparations from intact adult male rats had the highest specific content and appeared to be more homogeneous on SDS gel electrophoresis, this form of cytochrome P-450 was used as antigen for rabbit immunizations. Antiserum produced from the rabbit was used for both double immunodiffusion and inhibition of testosterone hydroxylation studies. In the Ouchterlony double diffusion plates, antiserum reacted with all partially purified cytochrome P-450 preparations (both
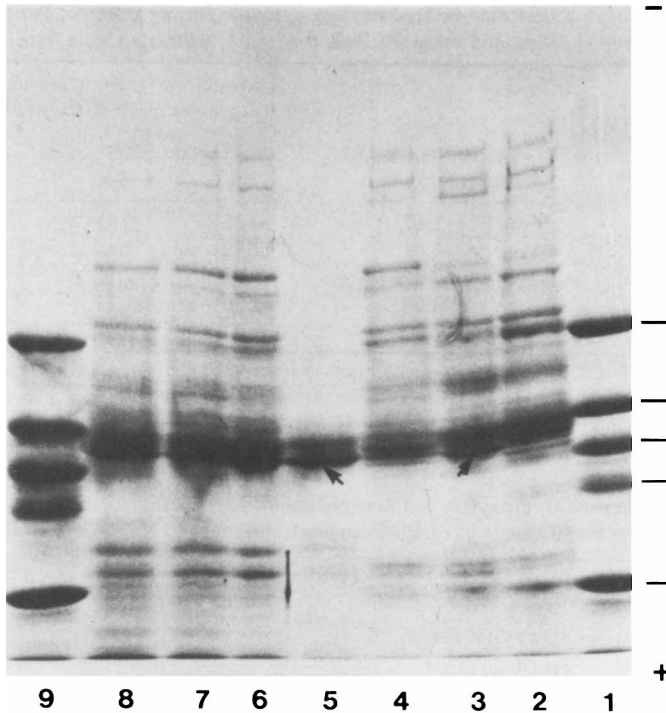
Fig. 1. Polyacrylamide gel electrophoretic pattern of partially purified cytochrome P-450 from neo-natally imprinted and nonimprinted rats. Tracks 1 and 9 represent protein standards (bovine serum al-bumin, 68K; glutamate dehydrogenase, 35K; ovalbumin, 45K; aldolase, 40K; chymotrypsinogen A, 25K). Tracks 2–5 represent 10 μg of protein each of the partially purified cytochrome P-450 from adult male castrated at birth, adult male castrated at four weeks, adult female and adult male, respec-tively. Tracks 6–8 represent 20 μg of protein each of the cytochrome P-450 from octylamino-Agarose I, hydroxyapatite I, and DEAE-cellulose fractions of the adult male rats (see Methods). Arrows indicate a 50,000-dalton protein that appeared in the neonatally imprinted rats. This protein is either absent (track 2) or in low concentrations (track 4) in the neonatally nonimprinted preparations.

neonatally imprinted and nonimprinted rats, see Fig. 2A). The major immunoprecipitant line suggests the homology of these cytochrome P-450 preparations. However, a minor immunoprecipitant line was also observed in adult male rats castrated at four weeks of age (well 1), adult male (well 2), and adult male castrated at birth (well 4), but appeared ab-sent in adult female (well 3). Upon immunoabsorption against partially purified cyto-chrome P-450 from neonatally nonimprinted rats (adult female and adult male castrated at birth), both the major and minor immunoprecipitant lines disappeared, suggesting the homology of the partially purified cytochrome P-450 between neonatally imprinted and nonimprinted rats (Fig. 2B).

The residual nonabsorbed antiserum was added to a microsomal testosterone hydroxyl-ation assay system (see Methods). As compared to samples added with preimmunized rabbit serum, the antiserum inhibited both testosterone 16α- and 7α-, but not 6β-hydroxylase activities (Table IV). The inhibition by antiserum was concentration-dependent and was sensitive to boiling for 5 minutes at 100°C. Immunoabsorption studies revealed that when antiserum was absorbed by partially purified cytochrome P-450 from neonatally nonim-printed rats, the residual nonabsorbed antiserum still possessed the ability to inhibit testo-
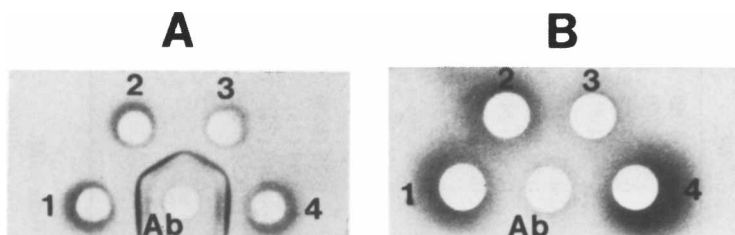
Fig. 2. Immunologic characterization of a neonatally imprinted form of cytochrome P-450. Ouchterlony double-diffusion experiments were performed by using plates containing 0.9% (w/v) Agarose and 0.2% (w/v) Emulgen 911. Plate A contained 12 μl of antiserum (Ab) in the center well and 12 μl of the partially purified cytochrome P-450 in the surrounding wells. The respective contents in the surrounding wells are: Well 1, 17 μM, adult male castrated at four weeks of age; well 2, 2.1 μM, adult male; well 3, 1.5 μM, adult female; and well 4, 2.9 μM, adult male castrated at birth. The center well (Ab) in plate B contained 12 μl of the residual antiserum absorbed against P-450 partially purified from neonatally nonimprinted rats (see also legend to Table IV). The surrounding wells contained partially purified P-450 as described above.

sterone 16α- and 7α- but not 6β-hydroxylase activities in a concentration-dependent manner similar to that of the nonabsorbed antiserum. In contrast, when antiserum was absorbed against the partially purified cytochrome P-450 from adult male rats, the residual antiserum lost its inhibitory activity against testosterone hydroxylation at either 16α-, 7α-, or 6β-position (Table IV).

DISCUSSION

We have established previously that the detergent-solubilized hepatic microsomal cytochrome P-450 from both neonatally imprinted and nonimprinted rats can be fractionated into four peaks (designated as peaks I, II, III, and IV) by DEAE-cellulose column chromatography [5]. Assessment of testosterone 16α-hydroxylase activity, a neonatally imprinted enzyme, in a reconstituted assay system revealed that this enzyme activity appeared in both peak II and III/IV and the activity in peak II accounted for the major neonatal androgen-imprinted differences in 16α-hydroxylase activity in the hepatic microsomes. The specific site of regulation by the neonatal androgen action with respect to testosterone 16α-hydroxylase activity resided in the cytochrome P-450 and appeared not to involve the modification of NADPH-cytochrome c reductase and/or lipids. The unique aspect of this regulation is that the neonatal androgenic stimuli irreversibly promotes the differentiation of certain hepatic drug or steroid-metabolizing enzyme systems during the critical period long after the initial hormonal stimuli were excreted.

In the present report, we have attempted to partially purify this sex-dependent and neonatally imprinted form(s) of cytochrome P-450 from both neonatally imprinted and nonimprinted rats. Biochemical and immunochemical data suggest that this form of cytochrome P-450 is imprintable in the male rats and is responsible for testosterone hydroxylation at 16α-position later on in adulthood.

Because of limited studies on isolation of hepatic cytochrome P-450 from untreated normal rats [16, 22], we have developed an isolation scheme, based on procedures established previously by several other laboratories [7, 16], to partially purify this form of hepatic cytochrome P-450 from untreated adult male rats. The partially purified cytochrome P-450s have specific contents ranging from 4.6–7.2 nmole/mg protein. This repre-

TABLE IV. Effects of Preimmunized, Unabsorbed, Absorbed, and Boiled Antisera on Hepatic Microsomal Testosterone Hydroxylase Activities

Group	Sample[a]	Immunoabsorption[b]	Volume (μl)	Testosterone hydroxylase activities[c] (nmole product/0.2 nmole P-450/15 min)		
				16α	7α	6β
I	Preimmunized serum	None	0	1.56 (100)	1.00 (100)	0.59 (100)
			37.5	1.39 (100)	0.83 (100)	0.68 (100)
			75	1.20 (100)	0.84 (100)	0.55 (100)
			150	0.58 (100)	0.45 (100)	0.41 (100)
II	Antiserum	None	0	1.37 (88)	0.94 (94)	0.79 (134)
			37.5	0.71 (51)	0.57 (69)	0.74 (109)
			75	0.11 (9)	0.11 (13)	0.68 (124)
			150	0.05 (9)	0.03 (6)	0.53 (129)
III	Antiserum	Absorbed by P-450 isolated from adult female and adult male rats castrated at birth	0	1.52 (97)	1.13 (113)	0.71 (120)
			37.5	0.89 (64)	0.75 (90)	0.54 (79)
			75	0.34 (28)	0.31 (37)	0.43 (78)
			150	0.08 (14)	0.15 (33)	0.38 (93)
IV	Antiserum	Absorbed by P-450 isolated from adult male rats	0	1.31 (84)	0.67 (67)	0.70 (119)
			37.5	1.34 (96)	0.86 (104)	0.53 (78)
			75	0.96 (80)	0.75 (89)	0.40 (73)
			150	0.72 (124)	0.49 (109)	0.34 (83)
II	Antiserum (boiled at 100°C for 5 min)	None	100	0.87	0.48	0.81
III	Antiserum (boiled at 100°C for 5 min)	See above, Group III	100	0.86	0.39	0.49

[a]Preimmunized serum (group I) and antiserum (group II) were obtained by diluting the respective serum with two volumes of buffer W.
[b]Immunoabsorption was achieved by mixing the antiserum with either one volume each of the partially purified cytochrome P-450s from adult female and adult male rats castrated at birth (group III) or one volume of the partially purified cytochrome P-450s from adult male and one volume of buffer W (group IV). The mixtures were allowed to interact overnight (12–16 hours) at 2°C. The residual unabsorbed antiserum was obtained by centrifugation at 20,000g for 10 minutes.
[c]Testosterone hydroxylase activities were assayed by first incubating microsomes with either preimmunized, unabsorbed, absorbed, or boiled antiserum at room temperature for one hour. The reaction was then initiated by the addition of other ingredients and allowed to proceed for 15 minutes at 35°C (see Methods). Numbers in parentheses represent the percent of control preimmunized activity.

sents a tenfold purification of cytochrome P-450 from hepatic microsomes [3, 15]. The partially purified cytochrome P-450 preparations consist of 23–36% cytochrome P-450 on a weight basis, assuming the specific content of the purified P-450 to be 20 nmole/mg protein. However, the purity may be underestimated, considering the possibility that the heme moiety may be dissociated from its apoprotein during purification. Although the purities of the present preparations are less than that reported previously by several other groups of investigators who have isolated cytochrome P-450 from phenobarbital, 3-methylcholanthrene, or 2,3,6,7-tetrachlorodibenzodioxin (TCDD)-induced rats [17, 18] or rabbits [19, 20], the specific contents of our preparation are comparable to others who attempted to purify cytochrome P-450 from untreated adult rats [16] or rabbits [20, 21]. More recently, however, cytochrome P-450s were purified from hepatic microsomes of untreated rats [22] or rabbits [23, 24] with higher specific contents.

Since antisera prepared against partially purified cytochrome P-450 from adult male rats reacted with other partially purified cytochrome P-450 from either nonimprinted or imprinted rats in double diffusion plates, these results suggest homology between cytochrome P-450 partially purified from neonatally imprinted and nonimprinted rats. This conclusion is further supported by the immunoabsorption data where the visualizable immunoprecipitable material in the antiserum can be completely removed by partially purified cytochrome P-450 from neonatally nonimprinted rats. Interestingly, the residual antiserum is still capable of inhibiting both testosterone 16α- and 7α-hydroxylase activities in a concentration-dependent manner. These data taken together suggest that the neonatally imprintable form of cytochrome P-450 only accounts for a small fraction of the total bulk of cytochrome P-450 in the partially purified P-450 preparation from the adult male rats. Although cytochrome P-450 with a molecular weight of approximately 50,000 daltons exhibits imprinting characteristics, the immunochemical data do not preclude the possibility that proteins other than the 50,000-dalton species may be neonatally imprinted. This is especially true when hemoproteins are not purified to homogeneity. In this context, it is interesting to note that the broad substrate specificity of cytochrome P-450 may be attributable to a large number of cytochrome P-450s. The number of forms of cytochrome P-450 may be as many as the number of immunoglobulins [25].

Greengard [26] has reviewed many examples of enzyme differentiation. These enzyme systems appear to differentiate in clusters and are regulated by endogenous hormones during the perinatal period. Einarsson et al [2] has suggested that certain steroid-metabolizing enzyme activities may be imprinted prepubertally but stimulated postpubertally by gonadal hormones. The ontogeny of microsomal testosterone hydroxylase activities has been reported previously by Conney et al [27]. The imprintable testosterone 16α-hydroxylase activity in the intact male rats is very low at birth, but increases markedly with concomitant loss of 7α-hydroxylase activity during the pubertal period [2, 27]. Results of the present study provide additional data to support the concept that there may be an "imprintable cluster" of cytochrome P-450 that may be formed in the hepatic microsomes in the normal course of development. Under the regulation by gonadal hormones during the neonatal period, this imprinted form(s) of cytochrome P-450 may be responsible for certain male characteristics of steroid and drug metabolism. The nature of imprinting by gonadal hormones in normal development may be unique and cannot be substituted by other chemicals such as certain arylhydrocarbons [Guenthner and Nebert, unpublished observation]. In this regard, it should be noted that the consequence of modifying normal pattern of imprinting by chemicals could pose long-term adverse effects on the developing mammals.

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